

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



F1

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification ⁵ : C12Q 1/68</p>	<p>A1</p>	<p>(11) International Publication Number: WO 94/03633 (43) International Publication Date: 17 February 1994 (17.02.94)</p>
<p>(21) International Application Number: PCT/AU93/00394 (22) International Filing Date: 2 August 1993 (02.08.93) (30) Priority data: PL 3893 .. 31 July 1992 (31.07.92) AU (71) Applicant (for all designated States except US): GARVAN INSTITUTE OF MEDICAL RESEARCH [AU/AU]; St Vincents Hospital, 384 Victoria Street, Darlinghurst, NSW 2010 (AU). (72) Inventors; and (75) Inventors/Applicants (for US only) : MORRISON, Nigel, Alexander [AU/AU]; 7 Prince Edward Circle, Page-wood, NSW 2035 (AU). EISMAN, John, Allen [AU/AU]; 28 Chelmsford Avenue, Lindfield, NSW 2070 (AU). KELLY, Paul, James [AU/AU]; 131 Carrington Road, Waverley, NSW 2024 (AU).</p>		<p>(74) Agent: F B RICE & CO.; 28A Montague Street, Balmain, NSW 2041 (AU). (81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published With international search report.</p>
<p>(54) Title: ASSESSMENT OF TRANS-ACTING FACTORS ALLELIC VARIATION</p> <p>(57) Abstract</p> <p>The present invention provides a genetic test for assaying predisposition to and/or resistance to high rates of bone turn-over, development of low bone mass and responsiveness or otherwise to therapeutic modalities. This is a specific model for use in prediction of osteoporosis and likely response to preventive or therapeutic modalities. It is a general model of allelic variation in transcriptional regulators determining physiological set-points and thus susceptibility or resistance to certain pathophysiological states.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NE	Niger
BE	Belgium	GN	Guinea	NL	Netherlands
BF	Burkina Faso	GR	Greece	NO	Norway
BG	Bulgaria	HU	Hungary	NZ	New Zealand
BJ	Benin	IE	Ireland	PL	Poland
BR	Brazil	IT	Italy	PT	Portugal
BY	Belarus	JP	Japan	RO	Romania
CA	Canada	KP	Democratic People's Republic of Korea	RU	Russian Federation
CF	Central African Republic	KR	Republic of Korea	SD	Sudan
CG	Congo	KZ	Kazakhstan	SE	Sweden
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovak Republic
CM	Cameroon	LU	Luxembourg	SN	Senegal
CN	China	LV	Latvia	TD	Chad
CS	Czechoslovakia	MC	Monaco	TG	Togo
CZ	Czech Republic	MG	Madagascar	UA	Ukraine
DE	Germany	ML	Mali	US	United States of America
DK	Denmark	MN	Mongolia	UZ	Uzbekistan
ES	Spain			VN	Viet Nam
FI	Finland				

ASSESSMENT OF TRANS-ACTING FACTORS ALLELIC VARIATION

Field of the Invention

The present invention relates to a method of identifying allelic differences in trans-acting factors as a means of identifying individuals at risk to suffer from an adverse pathophysiological condition. The method of the present invention is particularly useful in assessing allelic variations in the vitamin D receptor gene and thereby predicting predisposition to low or high bone density. Moreover these variants could be used to predict long-term risk of osteoporosis as well as predicting response to different modalities of therapy. This effect is also a model of determination of predisposition to or resistance to other pathological or physiological variations due to other transcription factor gene variants and thus determining risk of disease and of response to therapy. Such transcriptional regulators could be, but not limited to, ligand-activated gene regulators such as the steroid/retinoid/thyroid hormone receptor gene family.

Background to the Invention

Vitamin D functions as a potent regulator of bone and calcium homeostasis as well as of cellular differentiation and replication in many target tissues. It acts as its dihydroxylated metabolite (1,25-dihydroxyvitamin D, or calcitriol) through the highly specific vitamin D receptor (1). This trans-acting transcriptional activator protein mediates calcitriol action in the regulation of the expression of target genes. Cloning the vitamin D receptor gene (2,3) showed it to be a member of the ligand-activated receptor superfamily that includes the receptors for steroid hormones (glucocorticoids, progesterone, estrogen, androgen, and mineralocorticoids) as well as thyroid hormones and vitamin A derivatives (4,5),

natural regulators of a large number of physiological and developmental processes. The mechanisms by which these receptor proteins mediate the regulation of gene expression has been a subject of intense research.

5 Rare overt mutations have been identified that compromise the function of receptors and that cause major functional disorders in humans and animals. For example, mutations in the vitamin D receptor gene, resulting in vitamin D-resistant rickets (6), and in
10 the androgen receptor, resulting in androgen insensitivity (7), have been reported, and in the estrogen receptor gene an infrequent natural polymorphism has been correlated with a high rate of spontaneous abortion (8). However, despite a wealth of
15 molecular information, little is known of the potential contribution of natural allelic variation in receptor genes to diversity of response to steroidal hormones in normal physiology and in disease states.

Osteoporosis is a major public health problem
20 among the elderly in most Western countries involving both enormous health care costs and debilitating long-term effects (Riggs NEJM). Since therapy of established osteoporosis remains far from satisfactory, prevention is the best choice. Preventative strategies
25 for osteoporosis must focus upon development of peak bone density in early adulthood and minimisation of age-related and postmenopausal bone loss. Evidence from twin and family studies have shown strong genetic effects on peak bone density that is modifiable by
30 hormonal factors, nutrition and life style (Kelly et al, OI). Twin studies have demonstrated that monozygotic twin pairs have a much greater concordance for axial and appendicular bone density than do dizygotic pairs. Analysis of these data indicated that
35 these genetic factors account for approximately 75% of the total variation on bone density. This effect has

been confirmed in mother-daughter pair studies. The present inventors analysed the potential mechanisms of this genetic effect in the twin model. The present inventors found that the genetic effect was apparent in certain biochemical indices of bone turnover, such as osteocalcin, a marker of bone formation. Moreover amongst dizygotic twins the higher osteocalcin level was associated with the lower bone density. The present inventors have also found that the genetic effect can be shown with equal strength in another marker of bone formation, i.e., procollagen type I C-terminal propeptide and less strongly in a marker of bone breakdown, collagen type I C-terminal telopeptide. Under normal circumstances bone formation and bone breakdown are tightly linked or "coupled" in the twin physiological process of bone turnover. Thus the somewhat surprising results from the twin studies indicate that the bone formation markers, as markers of bone turnover, predict bone density and that genetic regulation of bone turnover is the pathway of the strong genetic effect on bone density.

The cross-sectional data on bone density in twins suggested that a single gene or set of genes is responsible for the genetic effect on bone density. However, it was unknown how this effect is mediated and which gene or genes influence bone density. In recent studies, using restriction fragment length polymorphism, the present inventors have shown common allelic variation in the vitamin D receptor (VDR) locus predict osteocalcin, independent of age, sex or menopausal status (Morrison et al, PNAS). The vitamin D receptor gene, as the active hormonal form of vitamin D (1,25-dihydroxyvitamin D), is an important central regulator of bone and calcium homeostasis modulating intestinal calcium absorption, bone formation, recruitment of the bone resorbing cell (osteoclast) and

bone resorption per se as well as parathyroid hormone production and vitamin D's own activation in the kidney. Because of the likelihood that any alterations in the receptor for the active hormonal form of vitamin D could have such wide effects, the effect of these common VDR gene alleles on bone density was examined using a twin model. In the twin model, within-pair comparisons eliminate age and various cohort effects as confounders.

The studies have shown that common allelic variants in the VDR gene predict differences in bone density and account for 50-75% of the total genetic determination of bone density in the spine and hip.

It is believed that this is a clear example that genotypic variations in transcriptional regulators of genes encoding regulatory and/or structural proteins, determine physiological set-points and predisposition to pathophysiological states with implications for susceptibility to disease and for determining likely responses to therapy.

Accordingly in a first aspect the present invention consists in a method of assessing in an individual's predisposition to a pathophysiological state and/or likely response to therapy comprising analysing genotypic variations in transcriptional regulators of genes encoding regulatory and/or structural proteins.

In a second aspect the present invention consists in a method of predicting predisposition of an individual to low or high bone density comprising analysing allelic variation within the vitamin D receptor gene of the individual.

In a preferred embodiment of the present invention the analysis comprises restriction fragment length polymorphism using endonuclease digestion.

In a further preferred embodiment of the present invention a segment of the vitamin D receptor is amplified using polymerase chain reaction prior to endonuclease digestion.

5 In yet a further preferred embodiment of the present invention the endonuclease is selected from the group consisting of BsmI, ApaI, EcoRV and TaqI, and is most preferably BsmI.

10 In another preferred embodiment of the present invention the segment of the vitamin D receptor is amplified using a pair of primers selected from the group consisting of

5' - CAACCAAGACTACAAGTACCGCGTCAGTGA - 3'
and 5' - AACCAGCGGAAGAGGTCAAGGG - 3';
15 and 5' - CAGAGCATGGACAGGGAGCAAG - 3'
and 5' - GCAACTCCTCATGGCTGAGGTCTCA - 3'.

20 In a second aspect the present invention consists in a primer pair derived from the sequence of the VDR gene shown in Table 5 for use in amplifying a segment of the VDR gene using polymerase chain reaction, the segment including at least one of the BsmI, ApaI or TaqI cut sites as shown in Table 5.

In a preferred embodiment of this aspect of the present invention the primer pair is

25 5' - CAACCAAGACTACAAGTACCGCGTCAGTGA - 3' and
5' - AACCAGCGGAAGAGGTCAAGGG - 3', or
5' - CAGAGCATGGACAGGGAGCAAG - 3' and
5' - GCAACTCCTCATGGCTGAGGTCTCA - 3'.

30 The allelic makeup of other transacting factors which may be assessed include oestrogen and androgen receptors to determine risk of osteoporosis and/or ischaemic heart disease. The allelic makeup of the androgen receptor may be also used to assess risk and responsiveness to therapeutic intervention in skin
35 diseases. The allelic makeup of the glucocorticoid receptor and the retinoic acid receptor can be

determined to assess risk of osteoporosis. The allelic makeup of the mineralocorticoid receptor can be determined to assess risk of hypertension and the allelic makeup of proto-oncogenes can be determined to assess cancer risk. Tissue specific regulators can also be assessed to determine osteoporosis/cancer risk.

In order that the nature of the present invention may be more clearly understood, preferred forms thereof will now be described with reference to the following examples and figures in which:-

Figure 1 shows lumbar BMD differences in twin pairs according to vitamin D receptor alleles.

Figure 2 shows a map of the vitamin D receptor gene from exon 7 to the start of the 3' non-coding sequence of exon 9 showing the location of polymorphic restriction enzyme sites used in this study and the fragments amplified by PCR used to detect the RFLPs. Asterisk denotes polymorphic site while absence of the asterisk indicates an invariant site.

Figure 3 shows bone mineral density is different in VDR genotypes: female subjects. Data shows the population mean \pm standard error mean. p values are for the pairwise two-sided Students t-tests for the groups.

Figure 4 shows the genetic effect on bone mass at the lumbar spine is also apparent in males. Symbols are as for Figure 3.

Figure 5 shows age related regression of lumbar spine bone mineral density and intersection with the fracture threshold according to genotype.

Figure 6 shows age related regression of femoral neck bone mineral density and intersection with the fracture threshold according to genotype.

Figure 7 shows bone density differences between twin pairs with respect to zygosity and concordance for VDR alleles. Bone density in the lumbar spine and

proximal femur is expressed at the within pair percent difference in bone density in MZ and DZ twin pairs and according to whether the DZ twin pairs are concordant or discordant for the VDR. The DZ twins concordant for the VDR alleles are not significantly different from the MZ twins at any site, while the discordant DZ twins are significantly different (ANOVA) from both of these groups at each site. The difference between the total DZ group and those concordant for the VDR alleles compared with the MZ twins indicates that 75%, 48%, 59% and 90% of the genetic effect can be explained by the VDR alleles at the lumbar spine, femoral neck, Ward's triangle and trochanteric region of the proximal femur respectively. Genotype for another developmental transcriptional activator, retinoic acid receptor- α (21q7), did not predict Δ BMD at any site.

Figure 8 shows the difference in bone density between dizygotic twin pairs with respect to degree of discordance for VDR. The difference in bone density between twin pairs is plotted in three groups; 0 - complete concordance, 1 - one allele different, 2 - both alleles different. Panels A, B, C and D show the analyses for the VDR gene in the lumbar spine femoral neck, Ward's triangle and trochanteric region respectively. Regression analysis of this effect shows significant relationships at the lumbar spine ($p=0.0001$), Ward's triangle ($p=0.006$) and trochanteric region ($p=0.034$) and borderline at the femoral neck ($p=0.055$). Using the sib-pair variance approach, significant relationships were observed between the squared difference in bone density within each twin pair (Δ^2) and concordance for the VDR gene alleles at the lumbar spine, femoral neck and Ward's triangle and borderline at the trochanteric region of the proximal femur.

	Lumbar spine Δ^2	= 0.015+0.038 * Degree of discordance (r=0.43, p=0.001)
	Femoral neck Δ^2	= 0.015 + 0.016 * Degree of discordance (r=0.29, p=0.034)
5	Ward's triangle Δ^2	= 0.017 + 0.026 * Degree of discordance (r=0.34, p=0.01)
	Trochanteric region Δ^2	= 0.015 + 0.015 * Degree of discordance (r=0.27, p=0.05)

10 Figure 9 shows higher bone mineral density associated with the b allele of the VDR gene.

A. Lumbar spine bone mineral densities of dizygotic twin pairs discordant for Bsm-1 alleles (n=22) are plotted as twin and co-twin according to genotype. Lines connect bone mineral density values for a twin pair. In 21 of 22 pairs, the twin carrying extra presence of the site (b) alleles has the higher bone mass (open circles). A single twin pair (black circles) has the reverse situation.

20 B. Bone mineral density at the lumbar spine amongst unrelated premenopausal females according to VDR genotype. One of each (premenopausal female) MZ and DZ twin pair was randomly selected for this analysis and the numbers of individuals are shown for each group. It is clear that the BB genotype has a lower mean BMD at the lumbar spine while the bb group has the higher mean BMD. The magnitude of this effect can be appreciated in relation to the standard deviation of bone density in an age-matched population of about 0.11gm/cm² at each site. The mean \pm SE is plotted and significance of the difference between groups was calculated by ANOVA. The pair-wise comparisons were made by unpaired Student's-tests. the different groups were not significantly different for age, height or weight.

35 Figure 10 shows the results of calcitriol therapy in individuals of different genotype.

STUDY 1Methods

Two hundred eighty-eight subjects recruited for epidemiological studies of bone density were included in the study. All subjects were recruited from the Sydney metropolitan area, latitude 33°52'S, a region of high sunlight incidence. Ninety-one subjects of Caucasian British-Australian origin (United Kingdom and Irish background) with restriction fragment length polymorphism (RFLP) data for the three endonucleases had serum osteocalcin data available. None of the subjects was taking medication known to cause bone disease or influence osteocalcin levels. All subjects were Caucasian and had normal renal function as determined by serum creatinine.

Serum was collected in the morning after overnight fast, and none of the subjects was treated with calcitriol prior to venipuncture. Serum osteocalcin was determined by an in-house radioimmunoassay based on rabbit anti ovine osteocalcin (11). The normal range of osteocalcin found with this assay is 3-18ng/ml when purified ovine osteocalcin is used. Osteocalcin determinations were made prior to, and independently of, the RFLP analysis and the results were stored in a coded fashion.

DNA Analysis. The probe used to identify RFLPs was a 2.1-kilobase-pair fragment of the vitamin D receptor cDNA (3,18) covering the entire coding region but lacking the 3' untranslated portion of the mRNA. Extraction of DNA from blood and Southern blotting were done by standard methods. Restriction enzymes were obtained from Pharmacia-LKB and New England Biolabs and used according to the suppliers' specifications.

Statistical Methods. The relative association of the RFLP markers was assessed statistically for deviation from the null hypothesis of free association

by using contingency tables and χ^2 tests. The Statview-plus-graphics statistical package (Abacus Concepts, Berkeley, CA) run on a Macintosh SE/30 computer was used for analysis of variance (ANOVA).
5 Fisher's protected least-significant-difference (PLSD) test was used to assess the relationship between RFLP and serum osteocalcin. Significance levels quoted are for the initial F tests on the null hypothesis (no difference between the means) of the overall effect and
10 for the confidence level of the pairwise comparison of the continuous variable means of each categorical (RFLP) class.

Each RFLP marker system was considered separately for its association with osteocalcin serum
15 concentrations by ANOVA comparing categorical classes (RFLPs) against the continuous variable (osteocalcin). The osteocalcin values (ng/ml) were not normally distributed, and so nonparametric analysis was performed as well as logarithmic transformation as $\ln(1$
20 + osteocalcin).

Results

Two previously unreported frequent RFLPs (detected by Bsm I and EcoRV) were found by using the vitamin D receptor cDNA probe, in addition to a previously
25 reported RFLP detected by Apa I (18). The RFLPs were coded as Aa (Apa I), Bb (Bsm I) and Ee (EcoRV), where the uppercase letter signifies absence of the site and lowercase signifies presence of the site. The Mendelian nature of the RFLPs was verified by family
30 studies (data not shown). The frequencies of these RFLPs in 266 unselected volunteers unrelated to this study are shown in Table 1. The genotypes of 182 individuals were assessed with all three RFLPs (Table 2). They demonstrated a strong degree of
35 coassociation, indicating linkage disequilibrium at this locus. The RFLPs were highly associated such that

AA was found with BB and EE at frequencies of 83% and 92%, respectively; correspondingly, aa was found with bb and ee at frequencies of 61% and 72%, respectively. The subsequent functional analysis does not depend on haplotyping; however, only two of a possible eight haplotypes are needed to account for 53.2% of the test population. The apparent homozygotes define the most frequent possible haplotypes as a b e and A B E (Table 2).

TABLE 1

Frequencies of RFLP Alleles

Enzyme	Allele 1	Allele 2	n*
Apa I	A, 0.494	a, 0.506	256
Bsm I	B, 0.439	b, 0.560	182
EcoRV	E, 0.490	e, 0.510	255

* No. of individuals tested.

TABLE 2

Frequencies of RFLP Genotypes

Homozygotes	n*	Heterozygotes	n*
aa bb ee	26	Aa Bb Ee	72
AA BB EE	19	AA Bb EE	13
AA bb EE	2	aa Bb ee	8
aa BB ee	2	Aa Bb ee	8
		Aa bb Ee	7
		Aa bb ee	4

* No. of individuals per 182 tested with all three RFLPs (heterozygote classes with <4 individuals have been excluded).

TABLE 3
Osteocalcin Values and Vitamin D Receptor Alleles

RFLP	n	Median	Mean	SD	SE	Sig.1	Sig. 2	P value
Bsm 1								
BB	16	16.8	2.86	0.45	0.11	BBvsbb	BBvsBb	0.0001
Bb	46	8.9	2.12	0.58	0.09		0.00005	0.0001
bb	25	8.8	1.97	0.71	0.14			
Total n	87							
Apa 1								
AA	25	14.0	2.53	0.63	0.13	AAvsaa	AAvsAa	0.0023
Aa	45	9.3	2.18	0.59	0.09		0.04	
aa	20	7.5	1.83	0.78	0.18			
Total n	90							
ECORV								
EE	26	14.0	2.53	0.63	0.12	EEvsee	EEvsEe	0.0153
Ee	45	8.8	2.11	0.60	0.09		0.015	
ee	18	10.5	2.02	0.83	0.20			
Total n	89							

Osteocalcin values among 91 Caucasian subjects of British-Australian origin (United Kingdom and Irish background) were analyzed with respect to the three informative RFLPs. As the osteocalcin values were not normally distributed, they were logarithmically transformed prior to statistical analysis. Median, median of serum osteocalcin values; Mean, mean of log-transformed values [ln(osteocalcin + 1)]; n, number of subjects; SD, standard deviation; SE, standard error of the mean; Sig., significance (probability that such a difference could occur by chance) referring to the difference between the means of the homozygotes (Sig. 1) and to the difference between the homozygote (absence of RFLP site) and the heterozygote (Sig. 2). P value is for the F test on the overall effect.

SUBSTITUTE SHEET

TABLE 4

Distribution of Subjects with respect to
Age, Sex, and Menopausal Status with respect to RFLPs

	Bsm 1			Apa 1			EcoRV		
	BB	Bb	bb	AA	Aa	aa	EE	Ee	ee
No. of subjects	16	46	25	25	45	20	26	45	18
Age, years	52+14	50+14	44+14	51+15	50+15	45+12	49+14	50+15	46+12
No. female	16	38	20	22	36	18	23	38	15
Postmenopausal	10	18	7	13	17	6	10	22	7
Premenopausal	6	20	13	9	19	12	13	16	8
No. male	0	8	5	3	9	2	3	7	3

SUBSTITUTE SHEET

The relationship between RFLPs and serum osteocalcin was analyzed in the 91 normal subjects with serum osteocalcin data (Table 3). The distribution of this population with respect to age, sex, and menopausal status is shown in Table 4. Age was not significantly related to any RFLP genotype. The osteocalcin levels of the Bsm I BB group are significantly higher than those of the Bsm I bb group ($P=0.0001$). The other RFLPs show the same effect with highly significant P values for the Apa I allele system (AA versus aa, $P<0.0025$) and a weaker P value for the EcoRV RFLP (EE versus ee, $P=0.015$). With all three RFLPs the absence of restriction-site alleles (A, B, E) is associated with high osteocalcin levels and the presence of restriction-site alleles (b, a, and e, respectively) with low osteocalcin levels: BB, 16.8ng/ml; Bb, 8.9ng/ml; and bb, 8.8ng/ml (medians). Nonparametric statistical analysis (Kruskal-Wallis) of raw osteocalcin values gave essentially the same results as ANOVA: Apa I, $P = 0.0016$; Bsm I, $P=0.0001$; EcoRV, $P=0.0044$.

Since the Bsm I and Apa I RFLPs were the most predictive, the population was subdivided according to the nine possible combinations of these alleles. This produced a clear separation of the serum osteocalcin values according to genotype (Fig. 1). Since the weaker association of the EcoRV marker may be determined by its disequilibrium with the other markers, we examined the distribution of Apa I and Bsm I alleles and osteocalcin values within individuals with the EE genotype (Fig. 2). The Bsm I marker essentially dictated the inferred haplotypes and their associated osteocalcin values ($P=0.003$).

The genotype prediction of serum osteocalcin levels was maintained for Bsm I and Apa I when males ($n=14$) were excluded (Bsm I, $P=0.0001$; Apa I,

P=0.0034; ANOVA values for the overall effect). Menopause has been associated with an increase in osteocalcin values, with a wide variation in osteocalcin values being observed in the early postmenopausal years (19-21). Therefore the role of menopausal status was assessed by multiple regression analysis and analysis of covariance including age, menopausal status, and Bsm I genotype. Menopausal status was a weaker determinant of serum osteocalcin concentrations than Bsm I polymorphism ($r=-0.44$, $P<0.001$). Two-factor ANOVA yielded the same result; Bsm I, $P=0.0002$; menopausal status, $P=0.24$. Analyzing premenopausal and postmenopausal women separately did not alter the results, and genotype was a stronger predictor of menopausal status (Fig. 1).

STUDY 2

Materials and Methods

Subjects

Subjects were 535 unrelated volunteers (447 females and 88 males) who had enrolled in studies of the effect of genetics on bone density. The subjects were obtained from requests through the media in the Sydney metropolitan area. The mean ages of the subjects were 51.4 ± 13.8 yr (mean \pm SD; range 20-84yr) for females and 40.6 ± 16.0 yr (20-79yr) for males. Subjects in this analysis were of Caucasian British-Australian origin (United Kingdom and Irish background). Menopausal status was confirmed by the presence of elevated FSH and LH and low estradiol levels, with an absence of menses for at least 12 months. Subjects with a history of bone disease, illness, bilateral ovariectomy or drug use (including hormone replacement therapy) which could affect bone turnover and bone density were excluded from this study.

Bone Mineral Density Analysis

Bone mineral density (BMD), expressed as an area density in g/cm^2 , was measured in the lumbar spine (L2-4) and femoral neck using either dual photon absorptiometry or dual energy x-ray absorptiometry (Lunar DP3 or DEXA, respectively, Lunar Radiation NCo. Madison, WI) as previously described (Pocock et al. 1987).

DNA Analysis: PCR (Polymerase chain Reaction) and RFLP Analysis using Endonuclease Digestion

Blood was collected into heparin treated tubes and leukocytes separated by sedimentation through physiological saline solution in a clinical centrifuge. Purified leukocytes were lysed in leukocyte lysis buffer (10mM Tris-HCl, pH7.4, physiological saline and 0.5% w/v sodium dodecyl sulphate). Lysate was treated with proteinase K (Applied Biosciences, Palo Alto USA) at 50 $\mu\text{g}/\text{ml}$ for 2 hour at 65 Celsius. DNA was extracted by repetitive phenol chloroform solvent extraction as described in Maniatis et al. and ethanol precipitated prior. DNA was redissolved in TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) and quantitated by ultraviolet absorbance at 260 Nm.

The vitamin D receptor gene from exon 7 to the 3'-untranslated region was sequenced. The sequence is set out in Table 5.

Four oligonucleotide primers were synthesized to amplify the 3' flanking region of the VDR gene. Detection of the BsmI site was facilitated by amplifying a region spanning the site, with one primer originating in exon 7 (5'-CAACCAAGACTACAAGTACCGCGTCAGTGA-3') and the other in intron 8 (5'-AACCAGCGGAAGAGGTCAAGGG-3') producing a 825 base pair fragment. Detection of ApaI and TaqI sites was facilitated using a single amplification one

18

TABLE 5

1/5

Sequence Range: 1 to 2169

```

      10      20      30      40      50
      *      *      *      *      *
CAACC AAGAC TACAA GTACC GCGTC AGTGA CGTGA CCAAA GGTAT GCCTA GACTC
GTTGG TTCTG ATGTT CATGG CGCAG TCACT GCACT GGTTT CCATA CGGAT CTGAG
      primer 490 Exon 7 | Intron 7

      60      70      80      90      100      110
      *      *      *      *      *      *
CACCT CCTGG GGAGT CTTTT TCAGC TCCCA GATTC TGGCT CCACC CGTCC TGGGG
GTGGA GGACC CCTCA GAAAA AGTCG AGGGT CTAAG ACCGA GGTGG GCAGG ACCCC

      120      130      140      150      160
      *      *      *      *      *
TTTGG CTCCA ATCAG ATACA TGGGA GGGAG TTAGG CACCA ACAGG GAGAG AAGGG
AAACC GAGGT TAGTC TATGT ACCCT CCCTC AATCC GTGGT TGTCC CTCTC TTCCC

      170      180      190      200      210      220
      *      *      *      *      *      *
CGAGG GTCAG ACCCA TGGGG TTGGA GGTGG GTGGG CGGCT CCTCA GCTCT TGCCC
GCTCC CAGTC TGGGT ACCCC AACCT CCACC CACCC GCCGA GGAGT CGAGA ACGGG

      230      240      250      260      270
      *      *      *      *      *
GCAGT ACCTG GCCAT TGTCT CTCAC AGGCC GGACA CAGCC TGGAG CTGAT TGAGC
CGTCA TGGAC CGGTA ACAGA GAGTG TCCGG CCTGT GTCGG ACCTC GACTA ACTCG
      Intron 7 | Exon 8 primer 757

      280      290      300      310      320      330
      *      *      *      *      *      *
CCCTC ATCAA GTTCC AGGTG GGACT GAAGA AGCTG AACTT GCATG AGGAG GAGCA
GGGAG TAGTT CAAGG TCCAC CCTGA CTTCT TCGAC TTGAA CGTAC TCCTC CTCGT
      primer 758 | primer 456

      340      350      360      370      380
      *      *      *      *      *
TGTCC TGCTC ATGGC CATCT GCATC GTCTC CCCAG GTATG GGGCC AGGCA GGGAG
ACAGG ACGAG TACCG GTAGA CGTAG CAGAG GGGTC CATA CCGG TCCGT CCCTC
      Exon 8 | Intron 8

>SacI
|
      390      400      410      420      430      440
      *      *      *      *      *      *
GAGCT CAGGG ACCTG GGGAG CGGGG AGTAT GAAGG ACAA GACCT GCTGA GGGCC
CTCGA GTCCC TGGAC CCCTC GCCCC TCATA CTTCC TGTTT CTGGA CGACT CCCGG

      450      460      470      480      490
      *      *      *      *      *
AGCTG GGCAA CCTGA AGGGA GACGT AGCAA AAGGA GACAC AGATA AGGAA ATACC
TCGAC CCGTT GGA CTCCCT CTGCA TCGTT TTCCT CTGTG TCTAT TCCTT TATGG

      500      510      520      530      540      550
      *      *      *      *      *      *
TACTT TGCTG GTTTG CAGAG CCCCT GTGGT GTGTG GACGC TGAGG TGCCC CTCAC
ATGAA ACGAC CAAAC GTCTC GGGGA CACCA CACAC CTGCG ACTCC ACGGG GAGTG

```

19

2/5

560 570 580 590 600
 * * * * *
 TGCCC TTAGC TCTGC CTTGC AGAGT GTGCA GGC GA TTCGG TAGGG GGGAT TCTGA
 ACGGG AATCG AGACG GAACG TCTCA CACGT CCGCT AAGCC ATCCC CCCTA AGACT
 primer 834

Polymorphic site >BsmI

610 620 630 640 650 660
 * * * * *
 GGAAC TAGAT AAGCA GGGTT CCTGG GGCCA CAGAC AGGCC TGC GC ATTCC CAATA
 CCTTG ATCTA TTCGT CCCAA GGACC CCGGT GTCTG TCCGG ACGCG TAAGG GTTAT

670 680 690 700 710
 * * * * *
 CTCAG GCTCT GCTCT TGC GT GAACT GGGCT CAACA TTCCT GTTAT TTGAG GTTTC
 GAGTC CGAGA CGAGA ACGCA CTTGA CCCGA GTTGT AAGGA CAATA AACTC CAAAG

720 730 740 750 760 770
 * * * * *
 TTGCG GGCAG GGTAC AAAAC TTTGG AGCCT GAGAG ATGGT TCTGC CTATA TAGTT
 AACGC CCGTC CCATG TTTTG AAACC TCGGA CTCTC TACCA AGACG GATAT ATCAA

780 790 800 810 820
 * * * * *
 TACCT GATTG ATTTT GGAGG CAATG TGCAG TGACC CTTGA CCTCT TCCGC TGGTT
 ATGGA CTAAC TAAAA CCTCC GTTAC ACGTC ACTGG GAACT GGAGA AGGCG ACCAA

830 840 850 860 870 880
 * * * * *
 AGAGG TGAGA AGAGG GAGAA AAGGC CGAAG AGAAG TTATT GTGAC CTTGG GACAT
 TCTCC ACTCT TCTCC CTCTT TTCCG GCTTC TCTTC AATAA CACTG GAACC CTGTA

890 900 910 920 930
 * * * * *
 GATGT CCGTG ATGAG GTCCA AAGAG GGGCG GCCCT GCCTC AGCCT GTGCT AGTGG
 CTACA GCCAC TACTC CAGGT TTCTC CCCGC CGGGA CGGAG TCGGA CACGA TCACC

940 950 960 970 980 990
 * * * * *
 CCTGT GCCCA GGGAT GCTTT CCTGG ACTGG AGGCT CAAGG AATGG AGATG GCTCC
 GGACA CGGGT CCCTA CGAAA GGACC TGACC TCCGA GTTCC TTACC TCTAC CGAGG

1000 1010 1020 1030 1040
 * * * * *
 TCTAC CCCTG CCCAG CCAGC CTTCT CTCAT TCATT CATCC ACTTA GCAAC AATTT
 AGATG GGGAC GGGTC GGTCT GAAGA GAGTA AGTAA GTAGG TGAAT CGTTG TTAAA

>KpnI
 1050 1060 1070 1080 1090 1100
 * * * * *
 ATTGA GCACC TATTA GGTAC CAGGC ACTAT GCTAG GTACT GGGGT TCAGC AGCAA
 TAACT CGTGG ATAAT CCATG GTCCG TGATA CGATC CATGA CCCCC AGTCG TCGTT

3 / 5

	1550		1560		1570		1580		1590	
*	*	*	*	*	*	*	*	*	*	*
CAGGC	AGTGG	TATCA	CCGGT	CAGCA	GTCAT	AGAGG	GGTGG	CCTAG	GGGGT	GCTGC
GTCCG	TCACC	ATAGT	GGCCA	GTCGT	CAGTA	TCTCC	CCACC	GGATC	CCCCA	CGACG

21

Polymorphic site >Apa1 4/5

1600 1610 1620 1630 1640 1650
 * * * * *
 CGTTG AGTGT CTGTG TGGGT GGGGG GTGGT GGGAT TGAGC AGTGA GGGGC CCAGC
 GCAAC TCACA GACAC ACCCA CCCCC CACCA CCCTA ACTCG TCACT CCCC GGTCT

>Sac1 >Sau3A1
 | |
 1660 1670 1680 1690 1700
 * * * * *
 TGAGA GCTCC TGTGC CTTCT CTATC CCCGT GCCCA CAGAT CGTCC TGGGG TGCAG
 ACTCT CGAGG ACACG GAAGA GATAG GGGCA CGGGT GTCTA GCAGG ACCCC ACGTC
 Intron 8 | Exon 9

Absence of Taq site

GATCGAGGCC = Taq1+ allele

>Pst1
 1710 1720 1730 1740 1750 1760
 * * * * *
 GACGC CGCGC TGATT GAGGC CATCC AGGAC CGCCT GTCCA ACACA CTGCA GACGT
 CTGCG GCGCG ACTAA CTCCG GTAGG TCCTG GCGGA CAGGT TGTGT GACGT CTGCA
 Primer 466 primer 455 and

>Sau3A1
 |
 1770 1780 1790 1800 1810
 * * * * *
 ACATC CGCTG CCGCC ACCCG CCCCC GGGCA GCCAC CTGCT CTATG CCAAG ATGAT
 TGTAG GCGAC GGCGG TGGGC GGGGG CCCGT CGGTG GACGA GATAC GGTTC TACTA
 770 (reverse 455)

1820 1830 1840 1850 1860 1870
 * * * * *
 CCAGA AGCTA GCCGA CCTGC GCAGC CTCAA TGAGG AGCAC TCCAA GCAGT ACCGC
 GGTCT TCGAT CGGCT GGACG CGTCG GAGTT ACTCC TCGTG AGGTT CGTCA TGGCG
 primer 467

1880 1890 1900 1910 1920
 * * * * *
 TGCCT CTCCT TCCAG CCTGA GTGCA GCATG AAGCT AACGC CCCTT GTGCT CGAAG
 ACGGA GAGGA AGGTC GGAAT CACGT CGTAC TTCGA TTGCG GGGAA CACGA GCTTC
 primer 468

>Sau3A1
 |
 1930 1940 1950 1960 1970 1980
 * * * * *
 TGTCT GGCAA TGAGA TCTCC TGACT AGGAC AGCCT GTGCG GTGCC TGGGT GGGGC
 ACAA CCGTT ACTCT AGAGG ACTGA TCCTG TCGGA CACGC CACGG ACCCA CCCCC

1990 2000 2010 2020 2030
 * * * * *
 TGCTC CTCCA GGGCC ACGTG CCAGG CCCGG GGCTG GCGGC TACTC AGCAG CCCTC
 ACGAG GAGGT CCCGG TGCAC GGTCC GGGCC CCGAC CGCCG ATGAG TCGTC GGGAG
 primer 459

2040 2050 2060 2070 2080 2090

```

      *      *      *      *      *      *      *      *      *      *
CTCAC CCGTC TGGGG TTCAG CCCCT CCTCT GCCAC CTCCC CTATC CACCC AGCCC
GAGTG GGCAG ACCCC AAGTC GGGGA GGAGA CGGTG GAGGG GATAG GTGGG TCGGG

```

```

      2100      2110      2120      2130      2140
      *      *      *      *      *      *      *      *      *
ATTCT CTCTC CTGTC CAACC TAACC CCTTT CCTGC GGGCT TTTCC CCGGT CCCTT
TAAGA GAGAG GACAG GTTGG ATTGG GGAAA GGACG CCCGA AAAGG GGCCA GGGAA

```

```

2150..      2160
      *      *      *      *
GAGAC CTCAG CCATG AGGAG TTGC
CTCTG GAGTC GGTAC TCCTC AACG
primer 465

```

Primer underlined on top strand is a forward primer, those on the bottom strand are reverse primers.
Any pair wise combination of these primers or primers based on this and surrounding sequence can amplify the region by polymerase chain reaction.

primer in intron 8 (5'-CAGAGCATGGACAGGGAGCAAG-3') and the other in exon 9 (5'-GCAACTCCTCATGGCTGAGGTCTCA-3') producing a 740 base pair fragment (Fig. 2).

5 PCR was carried out in a volume of 20 ul containing 200ng genomic DNA, 20pmol of each primer, 200 uM dNTPs, 50mM KCl, 10mM Tris (pH8.3), 1.5mM, MgCl₂ and 1U Taq DNA polymerase (TOYOBO, Osaka, Japan). Each sample was subjected to 37 amplification cycles as follows: Step 1 - 3 min at 94°C, 1 min at 62°C, 2 min at 72°C; Step 2 to 6 - 20 sec at 94°C, 20 sec at 62°C, 1 min at 72°C, Step 7 to 36 - 5 sec, 5 sec, 30 sec respectively. Amplification regimes should be optimised for any particular thermal cycling device. A 10 ul aliquot of each PCR product was digested with 5 units of endonuclease BsmI at 65°C (New England Biolabs, MA USA), Apal at 37°C or TaqI* (Promega Co. Australia) at 65°C for 1 hour. A clone of an unrelated gene was used as an internal control for both BsmI and Apal digestion. For TaqI digestion, an invariant TaqI site in the PCR product itself was used as an internal control. The digested PCR products were separated on 1.2% (BsmI and Apal), or 2.0% (TaqI) agarose gels containing 0.5ug/ml ethidium bromide, 0.09M Tris-Borate and 0.002M EDTA, pH8.3 for 1 hr at 100V. EcoRI digested SPP1 marker (Bresatec Limited, Adelaide, Australia) was used as the size standard for all agarose gels. Due to the sequence of the relevant sites several other restriction enzymes can be used to detect these polymorphisms. BsmI site sequence from an invariant adjacent Stu-1 site; B allele AGGCCTGCGCATTCCC, b allele underlined G is an A. This sequence change can be detected with AclI, FspI, MstI, Fdi2, HinfI, HhaI and their isoschizomers. Sequence at the polymorphic Apal site ending in an adjacent invariant Pvu2 site is: A allele GAGGGGCCAGCTG, in

10
15
20
25
30
35

the a allele the underlined G is a T. The presence of the G can be detected by Ban2, Aoc2, Pss1, Pal1, Hae3, Cfr3I, Asu1, Sau96I, Eco0109I, Dra2, and isoschizomers. The presence of the T creates a polymorphisms for Ban1, and its isoschizomers. The sequence of the Taq1 polymorphism spanning invariant Hba1 to Hae3 sites is: T allele GCGCTGATTGAGGCC, in the t allele the underlined T is a C. This polymorphism can be also detected by Mbo1, Sau3A, Dpn1 and their isoschizomers.

Taq1* RFLP: We have previously reported that Bsm1 and Apal RFLPs in the vitamin D receptor gene predict serum osteocalcin levels. These polymorphic sites are located in the region of genomic DNA from exon 7 to the 3' untranslated region (3'-UTR). To characterize the differences between two common vitamin D receptor gene alleles (AB and ab), we have sequenced this region in homozygotes of genotypes AABB, aabb. We have identified a number of sequence differences, including 15 non coding changes. There is a single synonymous coding region change, a T for C in an isoleucine codon (ATT to ATC, isoleucine codons) in exon 9.

Statistical Analysis

Analysis of the variance (ANOVA) was performed using Statview+Graphics statistical package (Abacus Concepts, Berkeley, CA, USA) on a Macintosh SE/30 computer. Fisher's protected least-significant-difference (PLSD) test was used to assess the relationship between RFLP and the BMD, height, weight. Significance levels quoted are for the initial F tests on the null hypothesis (no difference between the means) of the overall effect and for the confidence level of the pairwise comparison of the continuous variable means of each categorical (RFLP) class. Students t-test was used for pairwise comparisons. Relationships of continuous and categorical variables were established by multiple

regression. Relationships between RFLP markers were established by contingency tables and Chi square.

Results

The frequencies of these three RFLPs in 535 subjects are shown Table 6. The RFLPs were coded as Bb (BsmI), Aa (ApaI) and Tt (TaqI), where the uppercase letter signifies absence of the site and lowercase signifies presence of the site. The frequencies of BsmI and ApaI RFLP are similar to that set out above (Table 1). RFLPs had a high degree of coassociation (Table 7). The AA genotype is highly associated with BB and tt at frequencies 92.7% and 95.3%, respectively; correspondingly, aa was found with bb and TT at frequencies of 61.6% and 65.3%, respectively. Comparing BsmI with TaqI RFLP, tt, Tt, and TT genotypes are highly associated with BB, Bb and bb at frequencies 95.5%, 95.1% and 96.4% respectively. Because the BsmI and TaqI results are so closely correlated, in subsequent discussions we have equated BsmI and TaqI results and will refer only to BsmI results.

The relationship between RFLPs and BMD at both LS and FN sites were analyzed in the 535 subjects. The distribution of this population with respect to age, height, weight, and menopausal status is shown in Table 8. Age, height, and weight were not significantly related to any RFLP genotype (Table 9). In females, mean LS BMD of the BB and AA group are 9.9% (1.017 vs 1.118) and 8.6% (1.049 vs 1.139) lower than those of the bb and aa groups respectively. The FN BMD of the BB and AA groups are also 5.6% and 5.3% lower than those of the bb and aa groups respectively. A heterozygote effect indicating co-dominance of alleles was also observed (Figure 3). Lower LS and FN BMD were associated with the absence of both restriction site alleles (BA). The differences of mean BMD at LS and FN between BBAA genotype and bbaa genotype was wider

Table 6

Frequencies of RFLPs in study population.

Genotype	N	Frequency %	Allele
BB	89	16.8	B=0.418
Bb	266	50.1	b=0.582
bb	176	33.1	
AA	133	25.5	A=0.512
Aa	268	51.3	a=0.488
aa	121	23.2	
TT	188	35.4	T=0.596
Tt	257	48.4	t=0.404
tt	86	16.2	

Table 7

RFLP markers have a high degree of coassociation. Bsm-1 genotypes tabulated with Apa-1 and Taq1 genotypes. n refers to number of individuals. Chi square value and p value reflect the rejection of the null hypothesis of no association between the markers.

Marker	BB	Bb	bb	total n
AA	78	44	11	133
Aa	5	206	56	267
aa	4	8	106	118
total n	87	258	173	518

$$\text{Chi}^2=428 \quad p=0.0001$$

Marker	BB	Bb	bb	total n
TT	1	14	173	188
Tt	5	249	3	257
a	83	3	0	86
total n	89	266	176	531

$$\text{Chi}^2=912 \quad p=0.0001$$

Table 8

Population characteristics of study group

Sex	Number
Males	88
Females	447
Premenopausal	185
Postmenopausal	262
Mean years since menopause \pm SEM	11.3 \pm 0.07

Mean values of anthropomorphic parameters in total subjects (\pm SEM).

Age (year)	49.6 \pm 0.6
Height (cm)	163.8 \pm 0.4
Weight (kg)	64.8 \pm 0.5

Table 9

Mean values of anthropomorphic parameters according to Bsm-1 genotype

Genotype	n	Age	Height	Weight
Females				
BB	75	50 \pm 2	162 \pm 1	62 \pm 1
Bb	216	51 \pm 1	161 \pm 1	63 \pm 1
bb	154	52 \pm 1	161 \pm 1	64 \pm 1
p value		0.6	0.3	0.9
Males				
BB	14	35 \pm 4	177 \pm 2	72 \pm 10
Bb	50	41 \pm 2	176 \pm 1	75 \pm 10
bb	22	42 \pm 4	176 \pm 2	74 \pm 14
p value		0.9	0.6	0.4

Notes: n refers to number, p is the value for the overall effect of genotype on the variable in question, derived from ANOVA. All p values indicate no significant differences in mean values between different genotypes.

(13.4%, 7.8% respectively) than those of BB and bb or AA and aa (Table 9).

The effect of genotype was assessed by multiple regression analysis of covariance including age(yr), menopausal status (year post menopause; YPM), height (cm), weight (kg) and Bsm1 genotype (BB=1, Bb=2, bb=3) in females, giving the equation; LS BMD (g/cm²) = 0.419+0.054 Bsm1 genotype -0.004age -0.994 YPM+0.02 weight + 0.004 height (n=425) r=0.58 R²=0.34,

	Bsm1	age	YPM	Weight	Height
p value	0.0001	0.0013	0.0001	0.0045	0.003
F-score	24.9	16.0	10.4	8.2	8.9
FN BMD (g/cm ²)=0.456+0.025 Bsm1 genotype -0.004 age -0.004YPM + 0.04 weight + 0.02 height (n=425) r=0.68, R ² =0.47					

	Bsm1	age	YPM	Weight	Height
p value	0.002	0.0001	0.0004	0.0001	0.022
F-score	9.6	41.7	12.8	35.2	5.3

Both lumbar spine and femoral neck BMD were negatively and independently correlated with the menopausal status, age, Bsm1 RFP was also correlated independently with BMD at LS and F in females. Male's results were as follows:

LS BMD (g/cm²)=1.039+0.058 Bsm1 genotype (n=85)
r=0.22 RS=0.05, p=0.038, F-score 4.9

FN BMD(g/cm²)=1.046 -0.003age (n=85)
r=0./32, R²=0.10, p=0.017, F-score 5.9

30 Intercept with the Fracture Threshold

A value of lumbar spine BMD, below which a heightened risk of osteoporotic fracture exists, was derived from a large cross sectional study in the city of Dubbo Australia. This value 0.97 gm.cm² is similar to a fracture threshold described from an American population. Clearly, if VDR genotype affects BMD and

subsequently osteoporosis susceptibility, a difference in the intercept of the age related change in bone mass and the fracture threshold should be apparent between genotypes. Figure 5 shows simple age related regression lines for female LS BMD of BB, Bb and bb genotypes intersecting the fracture threshold value. A comparison between BB and bb reveals a 10 year difference in the intercept (60.3 yr versus 71.1 year, respectively) with an intermediate value for the Bb heterozygotes (68.1 year). A similar result was apparent for the neck of femur (Figure 6) using a fracture threshold of 0.7 gm/cm^2 (BB, 66 years; Bb, 70 years; bb, 74 years).

STUDY 3

The effect of the common VDR gene alleles on bone density was examined using the twin model, in which within-pair comparisons eliminate age and various cohort confounders. 250 Caucasian twins were studied comprising 70 MZ and 55 DZ twin pairs, including 7 male MZ pairs and 6 male DZ pairs, aged between 17 and 70 years; MZ 45 ± 13 yrs and DZ 44 ± 11 yrs, mean \pm SD. Bone density was measured at the lumbar spine and proximal femur with a Lunar DP3 dual-photon absorptiometer (LUNAR Corporation, Madison, WI) or Lunar DEXA dual energy X-ray absorptiometry as previously described (Pocock et al 1987). All female twin pairs were concordant for menopausal status and if post menopausal, for years since menopause.

The VDR gene in the region bearing the polymorphic sites for the Bsm-1, Apa-1 and EcoRV sites previously shown to predict differences in bone turnover markers was sequenced. These sites are in the region of the gene from exon 7 to the 3'-UTR. None of the polymorphic sites was in the coding region or involved potential splice sites and the highly informative Bsm-1 site was found to arise from a G for A substitution in

intron 8. There was only one difference in the coding region between the two most common allelic forms. This included a T for C substitution in exon 9, changing ATT to ATC, without changing the encoded amino acid sequence (isoleucine). The DNA sequence flanking the Bsm-1 site was used in a polymerase chain reaction-based method to amplify a 2.1 - 2.2 kb fragment from exon 7 to exon 9 to facilitate genotyping of subjects. PCR amplification of leucocyte DNA was performed with a Corbett FTS-1 Thermal Sequencer (Corbett Research, Mortlake NSW, Australia) PCR instrument using primers

5'-CAACCAAGACTACAAGTACCGCGTCAGTGA-3' and

5'-AACCAGCGGGAAGAGGTCAAGGG-3' prior to

endonuclease digestion with Bsm-1 (New England Biolabs Inc, Gene Search, Brisbane, Australia). The presence of the Bsm-1 site cuts a 825 bp product to 650 bp and 175 bp fragments. A 4.7 kb plasmid with a single Bsm-1 site, which linearises with Bsm-1 digestion, was used as an internal control to avoid misassignment of the allelic forms due to partial digests.

From twin studies the within pair difference in BMD (Δ BMD%) at the lumbar spine and proximal femur was examined in relation to allelic variation in DZ twin pairs (Figure 7). In both regions this was significantly less in DZ twins concordant compared to those discordant for VDR gene alleles. The Δ BMD% for lumbar spine in the MZ twins was not significantly different from that in the DZ twins concordant for the VDR alleles, both of which were statistically different from those in DZ twins discordant for the alleles ($p < 0.0001$). Similar but weaker effects in the proximal femur are consistent with stronger environmental influences on bone density in this region. Limiting the analysis to premenopausal twins did not alter the results. Controlling for potential confounding by

anthropomorphic features of height and weight, VDR genotype remained the strongest predictor at the lumbar spine ($p=0.0002$) and the trochanteric region ($p=0.02$) although not at the neck region of the proximal femur.

5 In view of the previously demonstrated co-dominant effect of Bsm-1 alleles on bone turnover indices, we would expect a co-dominant effect on the bone mass trait with a linear relationship between the degree of difference in genotype and the difference in trait

10 within twin pairs (Figure 8). According to the sib-pair linkage analysis approach, a significant correlation between the squared difference in a trait and the proportion of identical genes within a sibling pair indicates genetic linkage. By this analysis the

15 VDR gene alleles were co-dominant at the lumbar spine and most sites in the proximal femur. Comparing the Δ BMD% with respect to degree of concordance for VDR alleles showed 1.5 to 2.5-fold greater within pair differences for the discordant twins (see Figures 7 and

20 8). In 21 of 22 dizygotic twin pairs discordant for the VDR alleles, the b allele was associated with higher bone density (Figure 9A). In premenopausal females (randomly selected as singletons from MZ and DZ twin pairs), the VDR bb genotype was also associated

25 with higher bone density (Figure 9B) while the BB genotype was associated with lower bone mass with a clear codominant effect between the alleles (Figure 9B).

These data demonstrate that the differences of VDR

30 gene alleles indicate a major proportion of the differences in bone density in a population of normal individuals. The BB, AA, EE and/or tt VDR genotypes are associated with low BMD in both females and males. VDR gene RFLPs genotypes are therefore useful predictors of

35 propensity to high bone turnover and low bone mass, physiological variability not only in peak bone mass

but also bone mass in later life in both females and males. Until now, the mechanisms of the genetic effects on bone density and bone turnover have been unclear. However, 1,25-dihydroxyvitamin D is a
5 enhancer of osteocalcin synthesis through the vitamin D responsive element in the promotor of the VDR gene (Morrison 1989 Science). The present inventors have also shown that common allelic variants of the VDR gene are associated with differences in the serum
10 osteocalcin levels. Moreover, these allelic variants of the VDR gene predict the difference in bone density between dizygotic twin pairs.

It is concluded that these VDR gene RFLP's are markers for physiological variability in bone mass in both females and males. The present inventors have found that BsmI RFLP correlated independently with BMD at LS and FN.

Table 10. Age and years since menopause (YSM) amongst twins. A, those DZ twins concordant and discordant for VDR gene alleles; B; individuals with differing alleles for the VDR gene. All values are expressed as means \pm SD.

A

	Concordant	Discordant
Age	41.1 \pm 10.6 n = 30	45.5 \pm 12.3 n = 23
YSM	4.4 \pm 3.6 n = 5	9.3 \pm 5.3 n = 7

B

	BB	Bb	bb
Age	44.0 \pm 12.8	43.6 \pm 13.4	45.7 \pm 11.3
YSM	9.0 \pm 2.5 n = 9	11.8 \pm 9.5 n = 12	9.8 \pm 7.8 n = 7

Table 11A. Correlation co-efficients between monozygotic and dizygotic twin pairs with dizygotic pairs further segregated into those concordant and discordant for vitamin D receptor genotype.

Variable	rMZ	rDZ	P	rDZ		P
				concordant	discordant	
Lumbar Spine	0.81	0.16	<0.0001	0.41	0.04	<0.001
Femoral Neck	0.79	0.43	<0.0008	0.44	0.41	NS
Ward's Triangle	0.83	0.51	<0.004	0.43	0.54	NS
Trochanteric	0.82	0.34	<0.0002	0.49	0.38	<0.006
Weight	0.80	0.42	<0.0004	0.36	0.45	NS

Notes: p; denotes the p value for the test that correlation coefficients are significantly different. In the twin model a significant difference between rMZ and rDZ is evidence for a genetic effect on the trait in question. In our comparison of DZ twin pairs with the same within-pair VDR genotype (DZ-concordant) or different within-pair VDR genotype (DZ-discordant), a significantly different correlation between rDZ-concordant and rDZ-discordant is supportive of a contribution of the VDR genotype to the genetic effect on the trait in question.

SUBSTITUTE SHEET

Table 11B. Within-twin pair proportional difference in bone mineral density according to zygosity and degree of discordance of the twin pair of alleles of the vitamin D receptor gene. Values of percentage differences are means \pm sem.

Variable	n	Lumbar spine	Femoral Neck	Ward's Triangle	Trochanteric region
MZ	69	6.0 \pm 0.7	8.1 \pm 0.9	10.0 \pm 1.2	9.3 \pm 1.0
DZ-all	55	11.9 \pm 1.4	12.1 \pm 1.4	15.9 \pm 2.0	13.5 \pm 1.7
DZ-concordant	33	7.5 \pm 1.3	10.2 \pm 1.5	12.4 \pm 2.0	9.7 \pm 1.7
DZ-discordant	22	18.5 \pm 2.5	15.1 \pm 2.8	20.6 \pm 3.8	18.7 \pm 3.0
Genetic effect		75%	48%	59%	90%
DZ-discordant: -1 allele	14	15.6 \pm 2.5	13.2 \pm 2.7	16.4 \pm 3.7	19.9 \pm 2.8
-2 alleles	8	22.0 \pm 5.2	17.9 \pm 5.9	28.5 \pm 8.2	16.4 \pm 7.0
DZ-discordant/ DZ-con		2.47	1.48	1.66	1.93
DZ-2 alleles/ DZ-con		2.93	1.75	2.30	1.71

35

SUBSTITUTE SHEET

Importantly, the homozygous BBAA or AAtt genotype are associated with low bone density, and mean BMD at the LS and FN site were about 12% and 8% lower in BBAA homozygotes compared with bbaa genotype in both females and males. These genotypic differences are important for later life, because these differences of BMD indicate a 10 year difference in the fracture threshold. These allelic differences provide a mechanism for the genetic effect on bone mass observed in twin studies and provide a simple genetic test of carrier status for low bone mass alleles. Identification of the vitamin D receptor genotype as an important determinant of bone mass may open new avenues for prevention and therapy for osteoporosis.

15 Demonstration of Differences in Response to Treatment in Different Gene Types.

Data described above have demonstrated that the VDR alleles described above are functionally different. It would therefore be expected that individuals of different genotype would exhibit different responses to treatment with calcitriol and/or analogues. This was confirmed by examining responses to calcitriol administration in 10 normal young females of each homozygous Bsm 1 genotype (BB and bb) and analysing responses to treatment in three markers of bone calcium metabolism; osteocalcin, parathyroid hormone and urinary calcium (see Fig. 10).

Osteocalcin serum levels were different ($p < 0.01$) at baseline in the BB and bb groups. The BB genotype again had the higher osteocalcin. After calcitriol treatment the bb group had a higher percent response from baseline than the BB group. Although the BB group had a lesser percent response, since they had a higher baseline osteocalcin, the total response was higher.

35 Parathyroid hormone is known to be repressed by calcitriol, however, the extent of repression by

calcitriol treatment was significantly different in the two genotypic groups. Parathyroid hormone was weakly repressed in the BB group and strongly repressed in the bb group, indicating substantial differences in the response of PTH to calcitriol therapy. Total urinary calcium excretion over the treatment period (area under the curve) was significantly higher in the BB group than the bb group indicating different calcium handling responses according to genotype. the reduced repression of parathyroid hormone in the face of calcitriol treatment, coupled with increased urinary calcium output indicates different calcium homeostatic mechanisms, compatible with mobilisation of skeletal calcium.

VDR and Other Conditions

The vitamin D receptor and vitamin D endocrine system are implicated in several other pathological and physiological states. Such differences in the vitamin D receptor gene, leading to different responses to endogenous calcitriol, exogenous calcitriol and therapy using vitamin D analogues, will also result in differences in progression and susceptibility to other disorders where a significant component of regulation is effected by calcitriol. Known examples of conditions and diseases where the vitamin D endocrine system and VDR mediated events occur include AIDS virus (HIV-1) replication, breast cancer cell proliferation, colonic cancer cell growth, keratinocyte differentiation, psoriasis cell replication and function, spermatogenesis, melanoma and other tumours.

As a result of the invention described herein, it is therefore obvious that functionally different alleles of the VDR could affect the susceptibility, progress, prognosis and therapeutic efficacy of various treatments, in such diseases and conditions where the vitamin D receptor and vitamin D endocrine system are known to regulate aspects of the disease process. While

these are examples of physiological and disease processes influenced by the vitamin D endocrine system, it is in no way exclusive of other processes influenced by the vitamin D endocrine system. Given the data
5 described herein, it is obvious that all physiological and disease processes known to be influenced by the vitamin D endocrine system, as described in a recent comprehensive review by Walters, M (newly identified actions of the vitamin D endocrine system; Endocrine
10 reviews, 13:719-764) and papers referred to therein, could be assessed and investigated in the way described herein, and that these could be influenced by the vitamin D receptor genotype and therefore the genotype of an individual will be of importance to the prognosis,
15 progression, susceptibility and treatment of all conditions and diseases in which vitamin D receptor and the vitamin D endocrine system are involved.

Irrespective of the physiological mechanism, these data have identified for the first time a gene involved
20 in the regulation of bone density. Importantly the magnitude of the effect is such that it explains the majority of the strong genetic effect on bone density and indeed more than half of the adjusted population variation in bone density. These findings, which will
25 allow earlier interventions in those at increased risk of osteoporosis, provide important insight into the mechanism of the wide population variance in bone density and open the way to development of novel specifically targeted therapies. This single gene with
30 pleiotropic transcriptional activities is a model for many pathophysiological processes previously considered subject to complex multi-factorial genetic regulation.

This study describes a functional definition of naturally occurring alleles of a trans-acting
35 transcriptional activator by correlation with the product of a target gene. The data also indicate that

the receptor allelic differences also relate to major differences in a target organ - i.e., bone density. This method of genetic analysis provides a paradigm for the investigation of the functional significance of natural allelic variation within the genes of the ligand-activated receptor superfamily, which can contribute substantially to a more complete understanding of the steroid hormone endocrine system. It is also applicable to the genes for trans-acting regulators of all kinds.

Genotypic variations in transcriptional regulators of genes encoding regulatory and/or structural proteins, determine physiological set-points and predisposition to pathophysiological states with implications for susceptibility to disease and for determining likely responses to therapy. These genotypic variants are a general model for use in the determination of disease risk and for choice of therapy in prevention and treatment.

As a specific example of this model, allelic variants in the vitamin D receptor gene determine bone turnover, bone mass and sensitivity to environmental factors. As such these variants are markers of risk of development of osteoporosis and indicate likely response to various modalities of therapy.

The inventors have identified RFLP markers that define functionally different vitamin D receptor alleles. The RFLPs herein described are physical markers that are linked to genetic phenomena. The inventors advise that it is now obvious that any other RFLP, physical marker, polymorphic sequence, or genetic effect detectable in the vitamin D receptor gene or flanking DNA, which is in linkage with the currently defined markers, could provide the same information content as the markers herein described, dependent on the extent of linkage between the markers defined herein

and any other such marker, consisting of RFLP, physical, polymorphic sequence, or genetic effect. The inventors thereby state that other markers, known or unknown, in linkage with the markers herein described, represent a
5 claimed usage of this invention.

.. It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific
embodiments without departing from the spirit or scope
10 of the invention as broadly described. The present
embodiments are, therefore, to be considered in all
respects as illustrative and not restrictive.

REFERENCES

1. Haussler, M.R. (1986) *Annu. Rev. Nutr.* 6, 527-562.
2. McDonnell, D.P., Mangelsdorf, D.J., Pike, J.W., Haussler, M.R. & O'Malley, B.W. (1987) *Science* 235, 1214-1217.
3. Baker, A.R., McDonnell, D.P., Hughes, M., Crisp, T.M., Mangelsdorf, D.J., Haussler, M.R., Pike, J.W., Shine, J. & O'Malley, B.W. (1988) *Proc. Natl. Acad. Sci USA* 85, 3294-3298.
4. Yamamoto, K.R.A. (1985) *Annu. Rev. Genet.* 19, 209-252.
5. Evans, R.M. (1988) *Science* 240, 889-895.
6. Marcelli, M., Tilley, W.D., Wilson, C.M., Griffen, J.E., Wilson, J.D. & McPhaul, M.J. (1990) *Mol. Endocrinol.* 4, (8), 1105-1116.
7. Hughes, M.R., Malloy, P.J., Kjeback, D.G., Kesterson, R.A., Pike, J.W., Feldman, D & O'Malley, B.W. (1988) *Science* 242, 1702-1705.
8. Lehrer, S., Sanchez, M., Song, H.K., Dalton, J., Levine, E., Saporetti, P., Thung, S.N. & Schachter, B. (1990) *Lancet* 355, 622-624.
11. Kelly, P.J., Hopper, J.L., Macaskill, G.T., Pocock, N.A., Sambrook, P.M. & Eisman, J.A. (1991) *J. Clin. Endocrinol. Metab.* 72, 808-813.
18. Faraco, J., Morrison, N.A., Shine, J. & Frossard, P. (1989) *Nucleic Acids Res.* 17, 2150.
19. Delmas, P.D., Stenner, D., Wahner, H.W., Mann, K.G. & Riggs, B.L. (1983) *J. Clin. Invest.* 71, 1316-1321.
20. Kelly, P.J., Pocock, N.A., Sambrook, P.N. & Eisman, J.A. (1989) *J. Clin. Endocrinol. Metab.* 69, 1160-1165.
21. Catherwood, B.D., Marcus, R., Madvig, P. & Cheung, A.K. (1985) *Bone* 6, 9-13.
22. Pocock NA, Eberl S, Eisman JA, et al. Dual-photon bone densitometry in normal Australian women; a cross-sectional study. *Med J Aust* 1987; 146:293-7.

23. Pocock NA, Sambrook PN, Hille N. et al. Assessment of spinal and femoral bone density by dual X-ray absorptiometry; comparison of lunar and hologic instruments. J Bone Min Res 1992; 7: 1081-4.
24. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1981) Molecular Cloning: a laboratory manual Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

CLAIMS:-

1. A method of assessing an individual's predisposition to low or high bone density, development of high or low bone turnover and/or responsiveness to therapy comprising analysing allelic variation in relation to the vitamin D receptor gene of the individual.
2. A method as claimed in claim 1 in which the analysis comprises restriction fragment length polymorphism using endonuclease digestion.
3. A method as claimed in claim 2 in which a segment of the vitamin D receptor is amplified using polymerase chain reaction prior to endonuclease digestion.
4. A method as claimed in claim 2 or claim 3 in which the endonuclease is selected from the group consisting of Bsm1, Apal, EcoRV, Taq1, and isoschizomers thereof.
5. A method as claimed in claim 4 in which the restriction endonuclease is Bsm1.
6. A method as claimed in any one of claims 3 to 5 in which the segment of the vitamin D receptor is amplified using a pair of primers selected from the group consisting of
 - 5' - CAACCAAGACTACAAGTACCGCGTCAGTGA - 3'
 - and 5' - AACCAGCGGAAGAGGTCAAGGG - 3';
 - and 5' - CAGAGCATGGACAGGGAGCAAG - 3'
 - and 5' - GCAACTCCTCATGGCTGAGGTCTCA - 3'.
7. A method as claimed in any one of claims 1 to 6 in which the segment of the vitamin D receptor gene analysed represents a variable portion of the vitamin D receptor or gene regions in linkage with at least one of the Bsm1, Apal, EcoRV and Taq1 cut sites.
8. A primer pair derived from the sequence of the VDR gene shown in Table 5 for use in amplifying a segment of the VDR gene using polymerase chain reaction, the segment including at least one of the Bsm1, Apal or

TaqI cut sites as shown in Table 5 or a gene segment in linkage with at least one of these cut sites.

9. A primer pair as claimed in claim 8 in which the primer pair is

- 5 5' - CAACCAAGACTACAAGTACCGCGTCAGTGA - 3' and
 5' - AACCAGCGGAAGAGGTCAAGGG - 3', or
 5' - CAGAGCATGGACAGGGAGCAAG - 3' and
 5' - GCAACTCCTCATGGCTGAGGTCTCA - 3'.

- 10 10. A method of assessing an individual's predisposition to a pathophysiological state and/or likely response to therapy comprising analysing genotypic variations in transcriptional regulators of genes encoding regulatory and/or structural proteins.

1/11

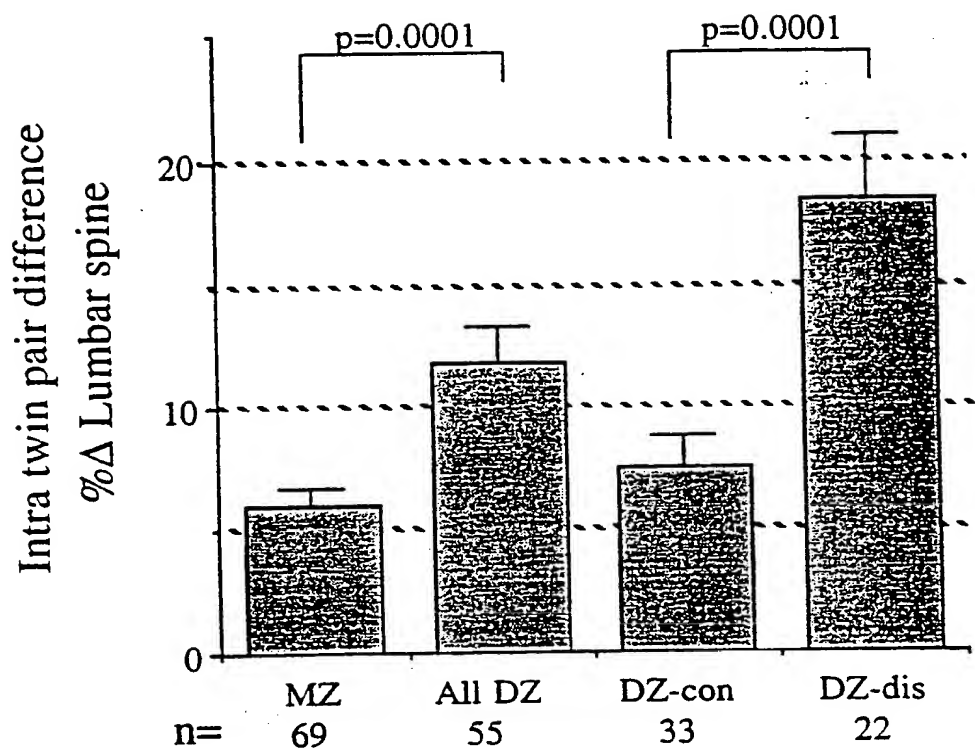


FIG. 1

SUBSTITUTE SHEET

2/11

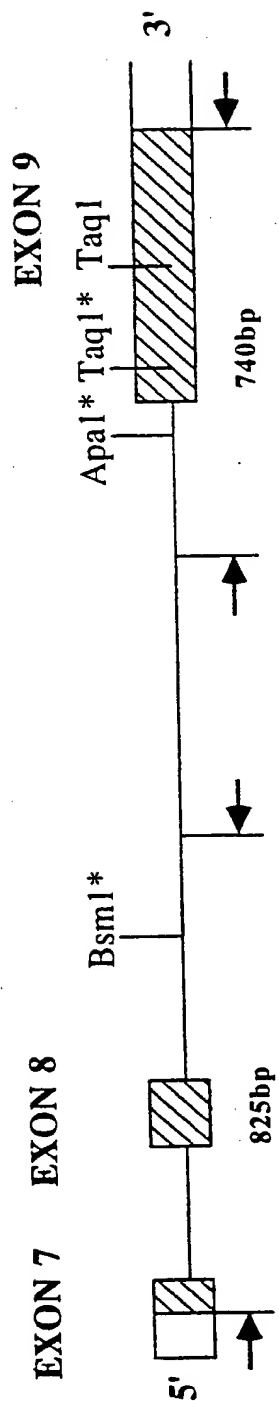


FIG. 2

3/11

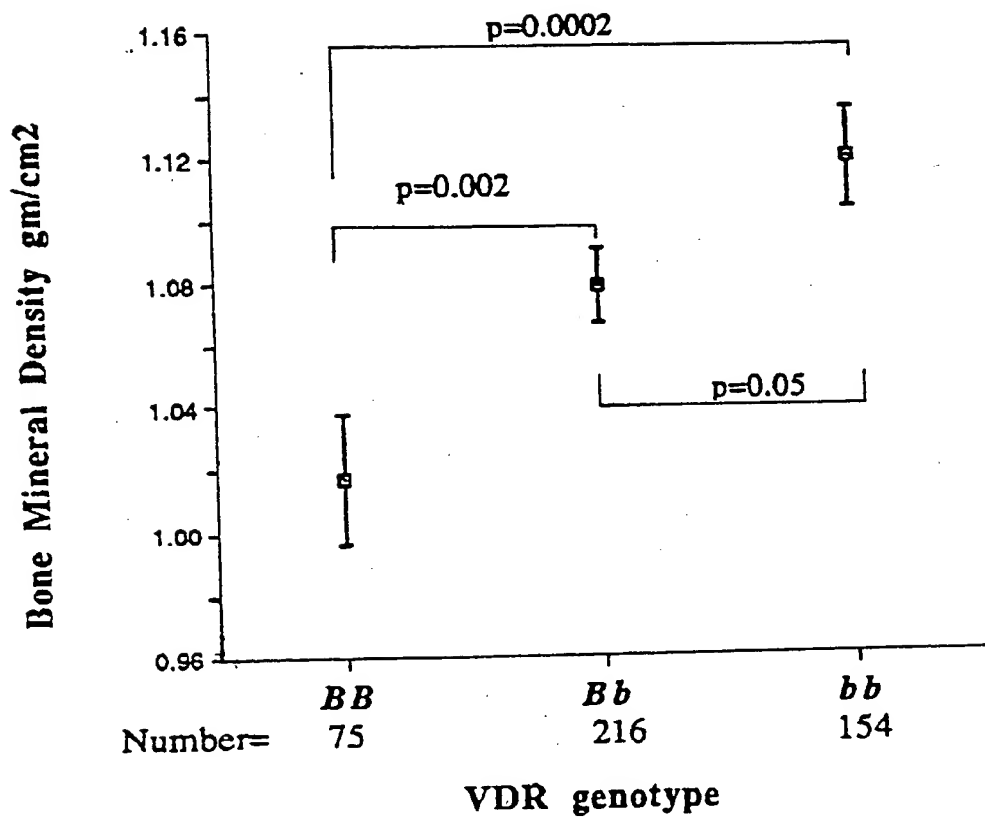


FIG. 3

SUBSTITUTE SHEET

4/11

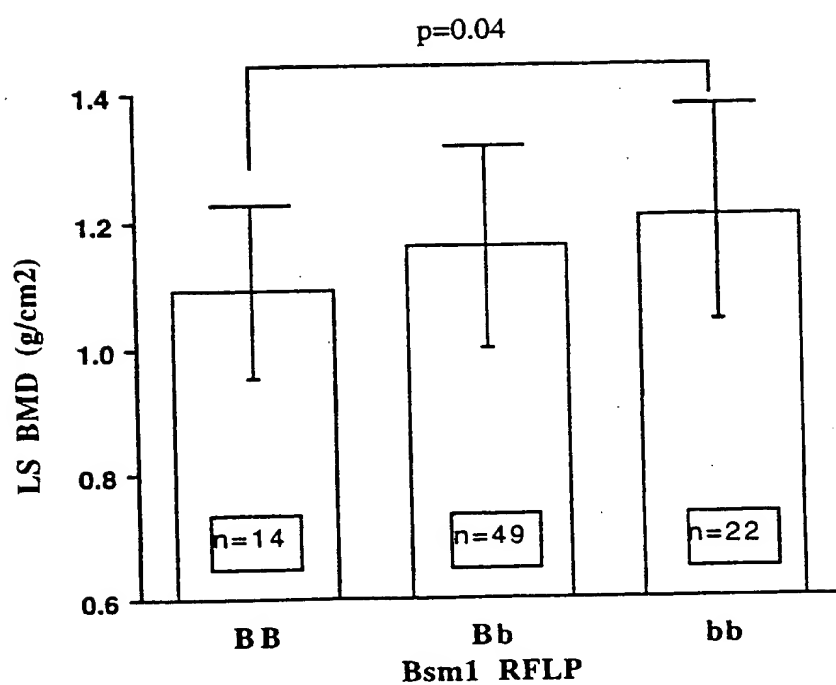
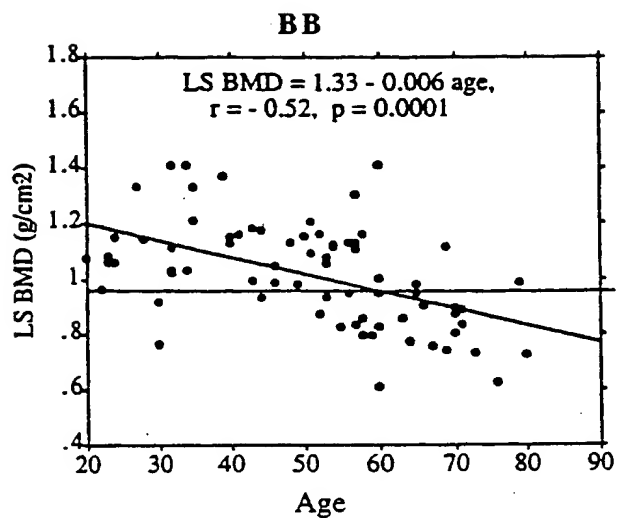


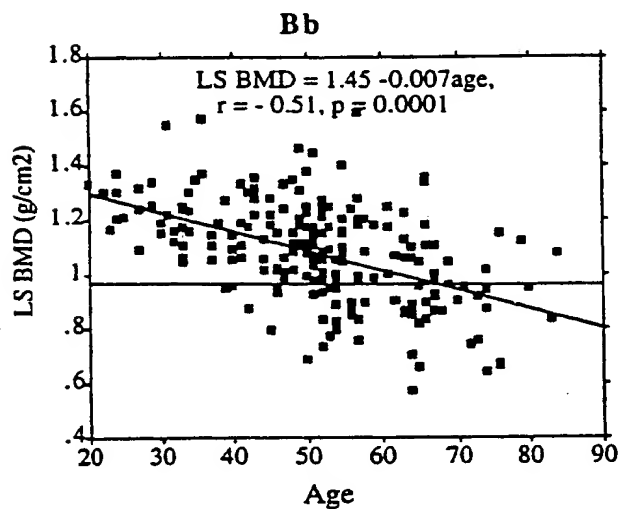
FIG. 4'

5/11



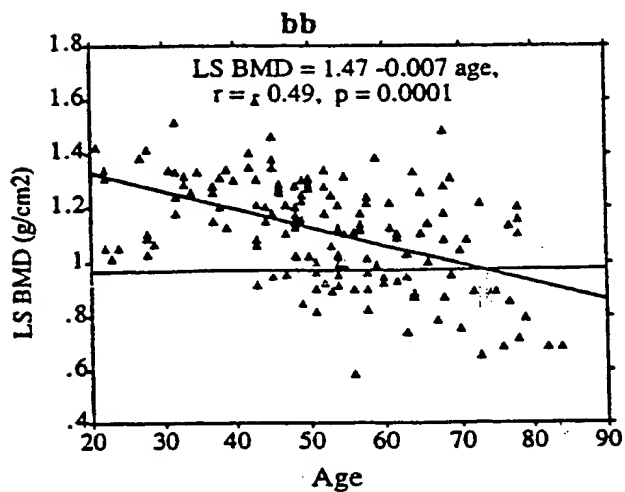
Intercept=60y

FIG. 5A



Intercept=68y

FIG. 5B



Intercept=71y

FIG. 5C

SUBSTITUTE SHEET

6/11

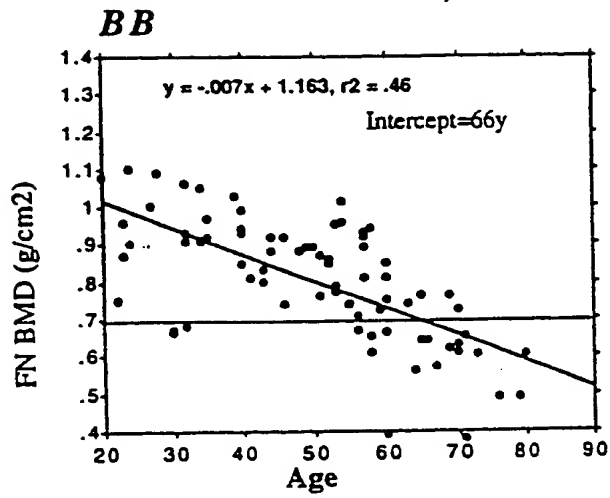


FIG. 6A

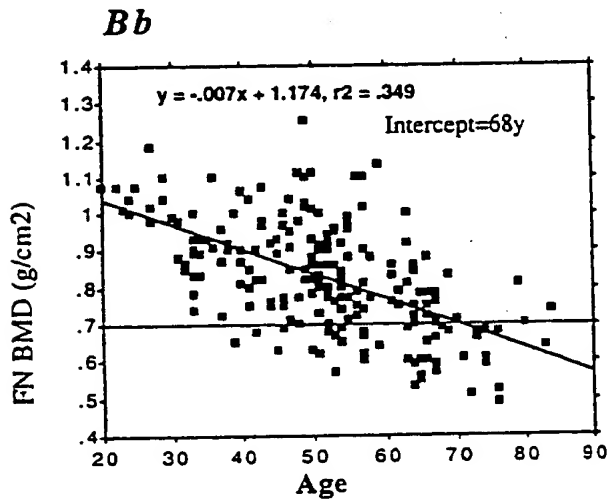


FIG. 6B

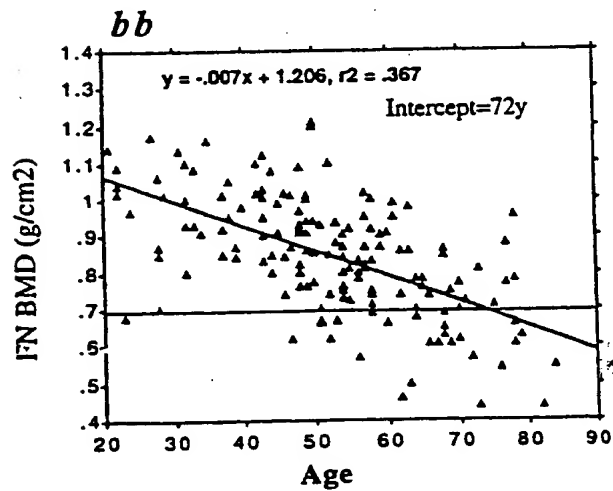
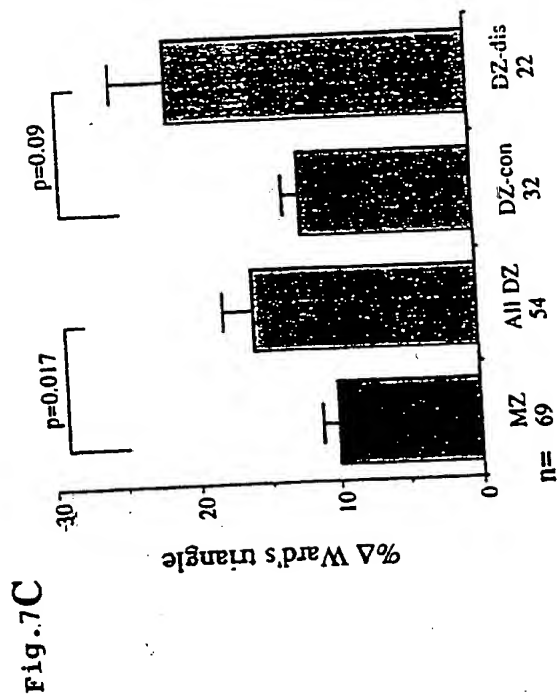
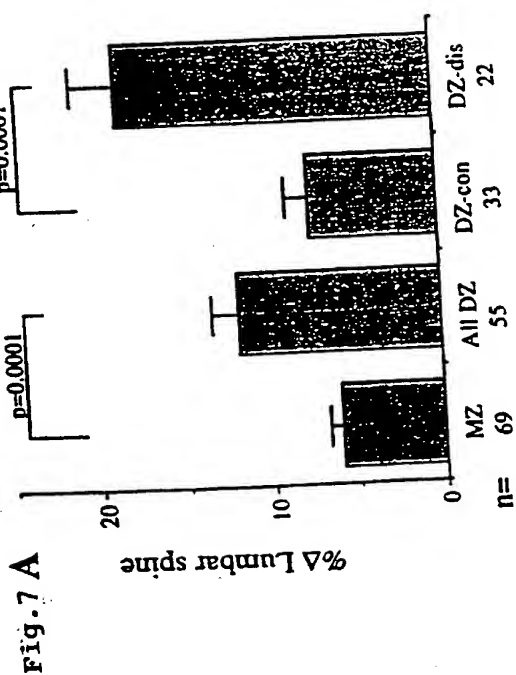
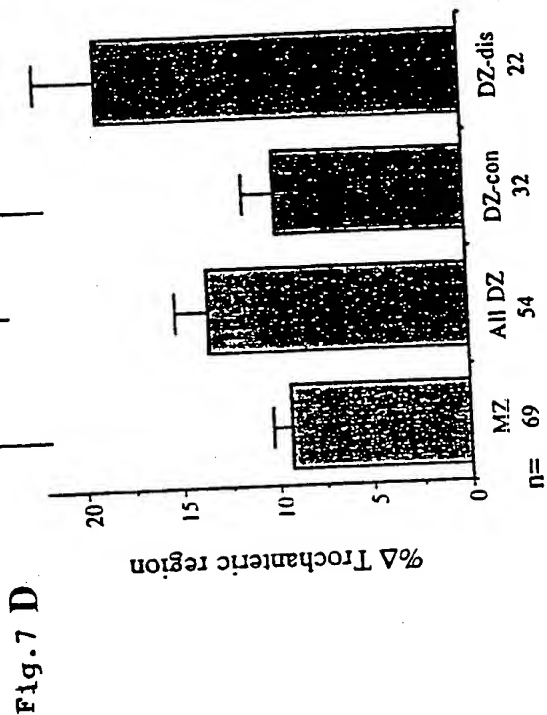
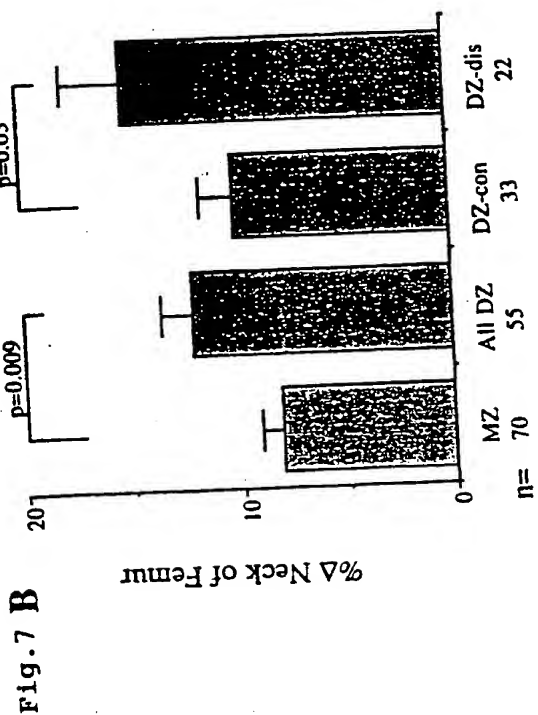


FIG. 6C

SUBSTITUTE SHEET

7/11



SUBSTITUTE SHEET

Fig. 8 A

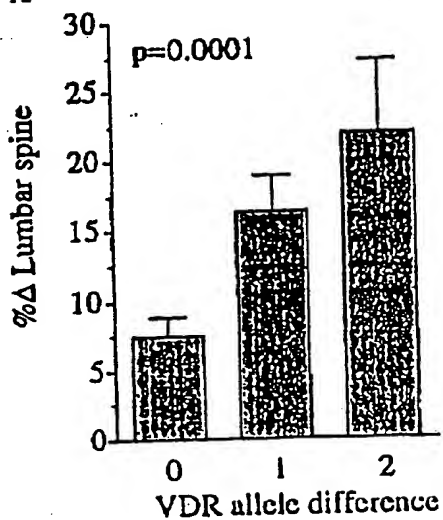


Fig. 8 B

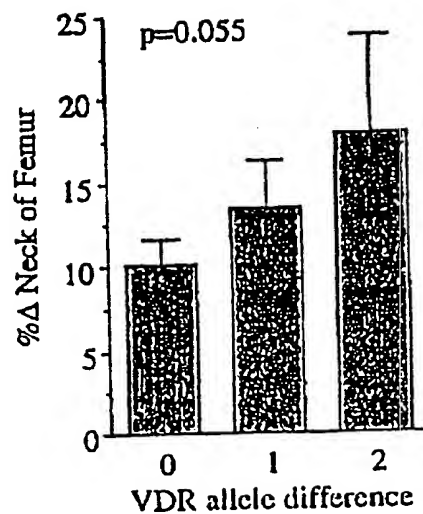


Fig. 8 C

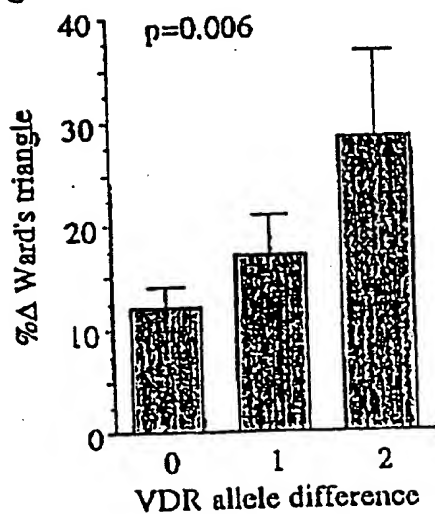
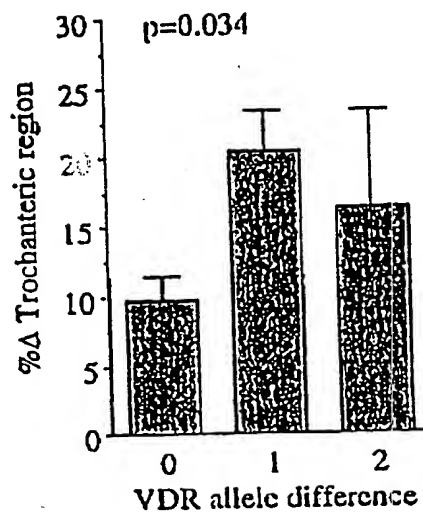


Fig. 8 D



SUBSTITUTE SHEET

9/11

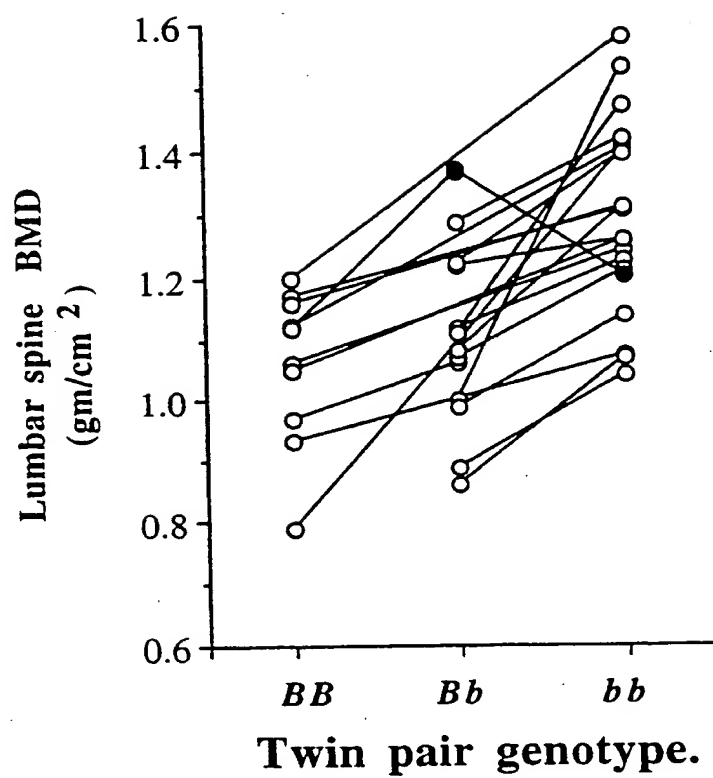


FIG. 9A

10/11

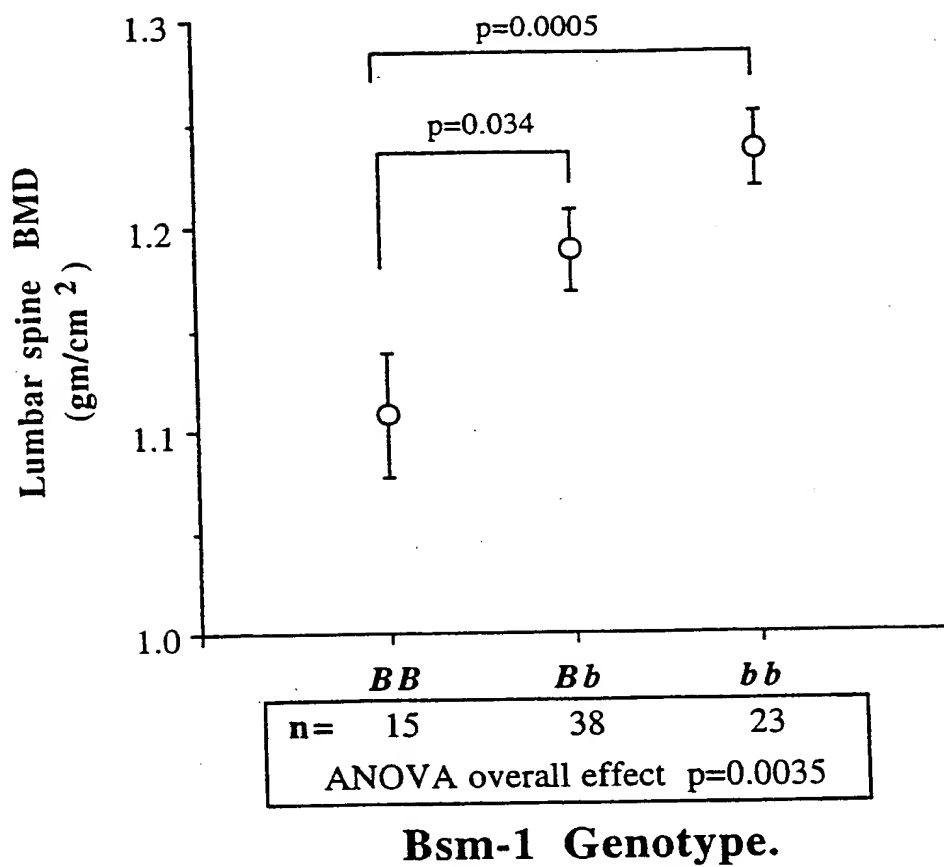


FIG. 9B

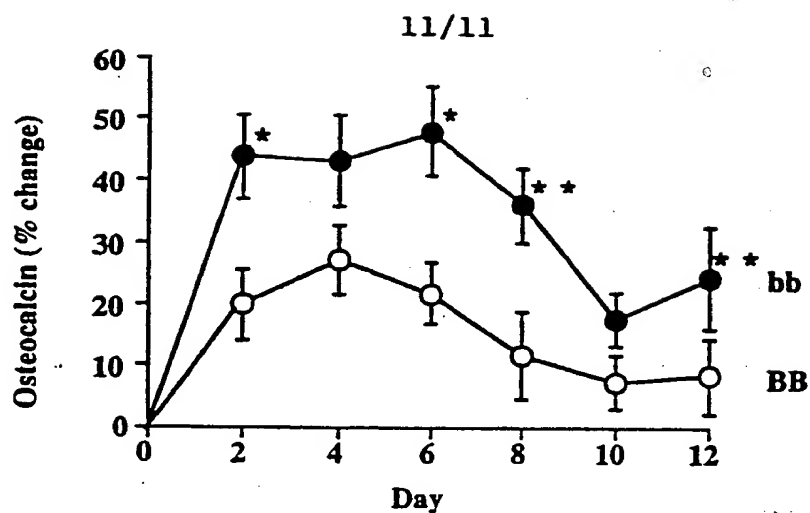


FIG. 10A

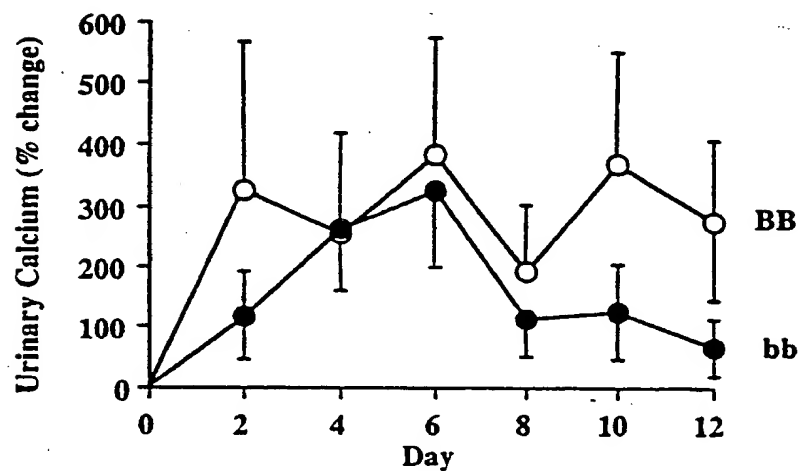


FIG. 10B

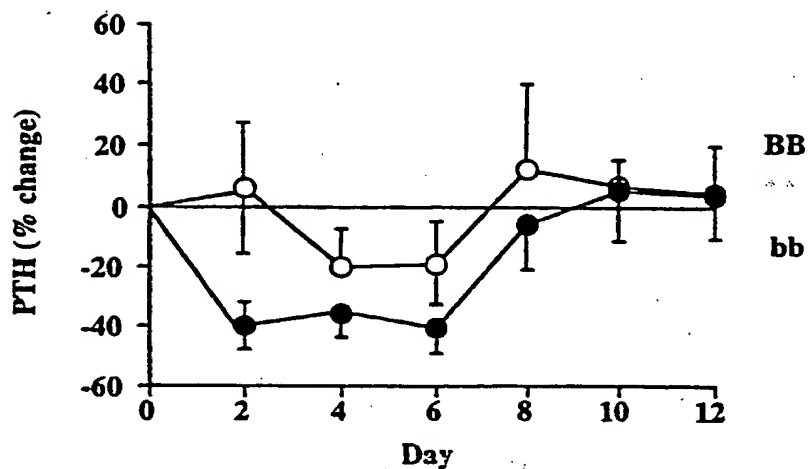



FIG. 10C

SUBSTITUTE SHEET

A. CLASSIFICATION OF SUBJECT MATTER Int. Cl. ⁵ C12Q 1/68 According to International Patent Classification (IPC) or to both national classification and IPC				
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC : C12Q, WPAT, CASM, BIOT, STN (See keywords below)				
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched IPC AU : C12Q 1/68 (020) + C12Q 1/68 (92-93)				
Electronic data base consulted during the international search (name of data base, and where practicable, search terms used) WPAT : Vitamin () D, vit () D, calciferol, VDR, bone or osteo: G01N 33/1C, C12Q 1/1C; VARIA: + TRANSCRIPTION: (S) REGULAT: CHEM ABS: AS ABOVE + 03/CC + 14/CC, BIOT: AS ABOVE, STN: PRIMERS OF CLAIM 6				
C. DOCUMENTS CONSIDERED TO BE RELEVANT				
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.		
P,X	Proc. Natl. Acad. Sci. USA. vol. 89, August 1992 Morrison, N.A. et al.; "Contribution of trans-acting factor alleles to normal physiological variability : Vitamin D receptor gene polymorphisms and circulating osteocalcin," pages 6665-6669.	1, 2, 4, 5, 7, 8, 10		
P,X	Proc. Natl. Acad. Sci. USA. vol. 90, March 1993, Bortell R et al; "Constitutive transcription of the osteocalcin gene in osteosarcoma cells is reflected by altered protein - DNA interactions at promoter regulatory elements", pages 2300-2304.	1, 10		
<div style="display: flex; justify-content: space-between;"> <div style="text-align: left;"> <input type="checkbox"/> Further documents are listed in the continuation of Box C. </div> <div style="text-align: left;"> <input type="checkbox"/> See patent family annex. </div> </div>				
<table style="width: 100%; border: none;"> <tr> <td style="width: 50%; vertical-align: top;"> <p>* Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </td> <td style="width: 50%; vertical-align: top;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p> </td> </tr> </table>			<p>* Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p>
<p>* Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p>			
Date of the actual completion of the international search 9 November 1993 (09.11.93)		Date of mailing of the international search report 18 NOV 1993 (18.11.93)		
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. 06 2853929		Authorized officer  CARMELA MONGER Telephone No. (06) 2832486		

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
Y	Molecular endocrinology, vo. 3(4), 1989, McDonnell D.P. et al; "Functional domains of the human vitamin D ₃ receptor regulate osteocalcin gene expression", pages 635-644.	2-9
X	Molecular endocrinology, vol. 6(4), 1992, Demay, M.B. et al; "Characterization of 1,25-Dihydroxyvitamin D ₃ receptor interactions with target sequences in the rat osteocalcin gene", pages 557-562.	1, 10
Y		2-9
X	AU, 17280/88 (Biotechnology Research Partners Ltd) 30 April 1987 (30.04.87).	10
X	AU,B, 57760/86 (598192) (Biotechnology Research Partners Ltd) 17 April 1985 (17.04.85).	10
X	AU 33223/93 (The Board of Trustees of the Leland Stanford Junior University), 17 December 1991 (17.12.91).	10
X	AU 62127/90 (Imperial Chemical Industries PLC) 7 September 1989 (07.09.89)	10
A	The Journal of Immunology, vo. 148(4) 1992, Shewey L.M. et al; "Differential expression of related HLA class II DQ β genes caused by nucleotide variation in transcriptional regulatory elements," pages 1265-1273.	
A	Proc. Natl. Acad. Sci. USA. vol. 89, Bortell R. et al., "Vitamin D - responsive protein - DNA interactions at multiple promoter regulatory elements that contribute to the level of rat osteocalcin gene expression", pages 6119-6123.	